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Regulation of Murine Cerebral Malaria Pathogenesis by CD1d-Restricted NKT Cells and the Natural Killer Complex

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Summary

NKT cells are specialized cells coexpressing NK and T cell receptors. Upon activation they rapidly produce high levels of interferon-γ (IFN-γ) and interleukin-4 (IL-4) and are therefore postulated to influence T_H1/T_H2 immune responses. The precise role of the CD1/NKT cell pathway in immune response to infection remains unclear. We show here that CD1d-restricted NKT cells from distinct genetic backgrounds differentially influence T_H1/T_H2 polarization, proinflammatory cytokine levels, pathogenesis, and fatality in the *P. berghei ANKA*/rodent model of cerebral malaria. The functional properties of CD1d-restricted NKT cells vary according to expression of loci of the natural killer complex (NKC) located on mouse chromosome 6, which is shown here to be a significant genetic determinant of murine malarial fatalities.

Introduction

About 2.5 million people die of severe *Plasmodium falciparum* malaria every year (World Health Organization, 1992). These fatalities are associated with a spectrum of discrete and overlapping disease syndromes of complex etiologies. Humans affected by malaria may suffer systemic, single-, or multi-organ involvement, including acute respiratory distress, coagulopathy, shock, metabolic acidosis, hypoglycemia, renal failure, pulmonary edema, and cerebral involvement including seizures and coma (White and Ho, 1992). Basic mechanisms controlling these processes are thought to be the site-specific localization of parasites by adherence to vascular endothelial markers such as the adhesin ICAM-1 (Berendt et al., 1989) and both local and systemic inflammatory responses arising from the action of cytokines.

Plasmodium berghei ANKA murine malaria has many features in common with the human disease and is thus an accepted model for certain important aspects of clinical malaria (Miller et al., 2002). It manifests a cytokine-dependent encephalopathy associated with upregulation of adhesins on the cerebral microvascular endothelium and attendant neurological complications (Grau et al., 1987, 1989; Jennings et al., 1997). Particularly in the developmental stages, the murine disease is accepted to reflect the cytokine-dependent inflammatory cascade contributing to cerebral and systemic involvement in humans. In the *P. berghei* ANKA cerebral malaria syndrome, causal roles for tumor necrosis factor-α (TNF-α) and IFN-γ in disease promotion have been established experimentally. Fatalities are abolished by passive transfer of monoclonal antibodies to IFN-γ (Grau et al., 1989) and polyclonal anti-TNF-α (Grau et al., 1987). Mutant mice lacking TNFα/β (Rudin et al., 1997a), lymphotoxin-α (LT-α) (Engwerda et al., 2002), IFN-γ (Rudin et al., 1997b), and TNF-receptor-2 (Lucas et al., 1997) have also been described as being resistant to disease. C57BL6 mice, genetically predisposed toward T_H1-dominated responses (Scott et al., 1989), are susceptible to the murine cerebral malaria syndrome, whereas BALB/c mice, with a genetically determined bias toward T_H2 responses (Scott et al., 1989), are resistant (de Kossodo and Grau, 1993). Thus, susceptibility to experimental cerebral malaria in mice is dependent on the production of proinflammatory and counterregulatory cytokines by T cells and the innate immune system (de Kossodo and Grau, 1993; Grau et al., 1986).

Because cytokine levels become elevated systemically very early during infection with *P. berghei*, it is reasonable to propose that nonconventional lymphoid populations capable of acting with accelerated kinetics may be the source of rapid and high level cytokine output involved in controlling pathology. The identity of such lymphoid sources, however, remains unclear. CD1d-restricted NKT cells are a novel T cell lineage with unusual features (Arase et al., 1992). Upon antigen-specific or polyclonal stimulation through the TCR, CD1d-restricted NKT cells produce large amounts of IL-4 and IFN-γ with rapid kinetics (Yoshimoto and Paul, 1994). They have therefore been postulated to influence T_H1/T_H2 differentiation of the acquired immune system (Porcelli, 1995). However, there is little evidence to date in support of this important proposition. Indeed, the functional significance of the CD1/NKT cell pathway in the immune response to infection is still controversial, as there are few reports to date of clear function for this population in infection models. CD1d-restricted NKT cells have been described to have a role in resistance to *Toxoplasma gondii* (Denkers et al., 1996) and *Leishmania major* (Ishikawa et al., 2000), and to increase susceptibility to *Salmonella* (Naiki et al., 1999) and *Listeria monocytogenes* (Emoto et al., 1995). In this study we sought to investigate the role of CD1-restricted NKT cells in the inflammatory cascade leading to *P. berghei*-mediated cerebral malaria. We found that the CD1d/NKT cell pathway differentially regulates T_H1/T_H2 polarization, malarial pathogenesis, and fatality according to the genetic background of the host. We further show that the natural killer complex (NKC) located on mouse chromosome 6 also regulates NKT cell function, malarial pathogenesis, and fatality. These loci account in part for the differential susceptibility of C57BL6 and BALB/c mice to the *P. berghei* cerebral malaria syndrome.

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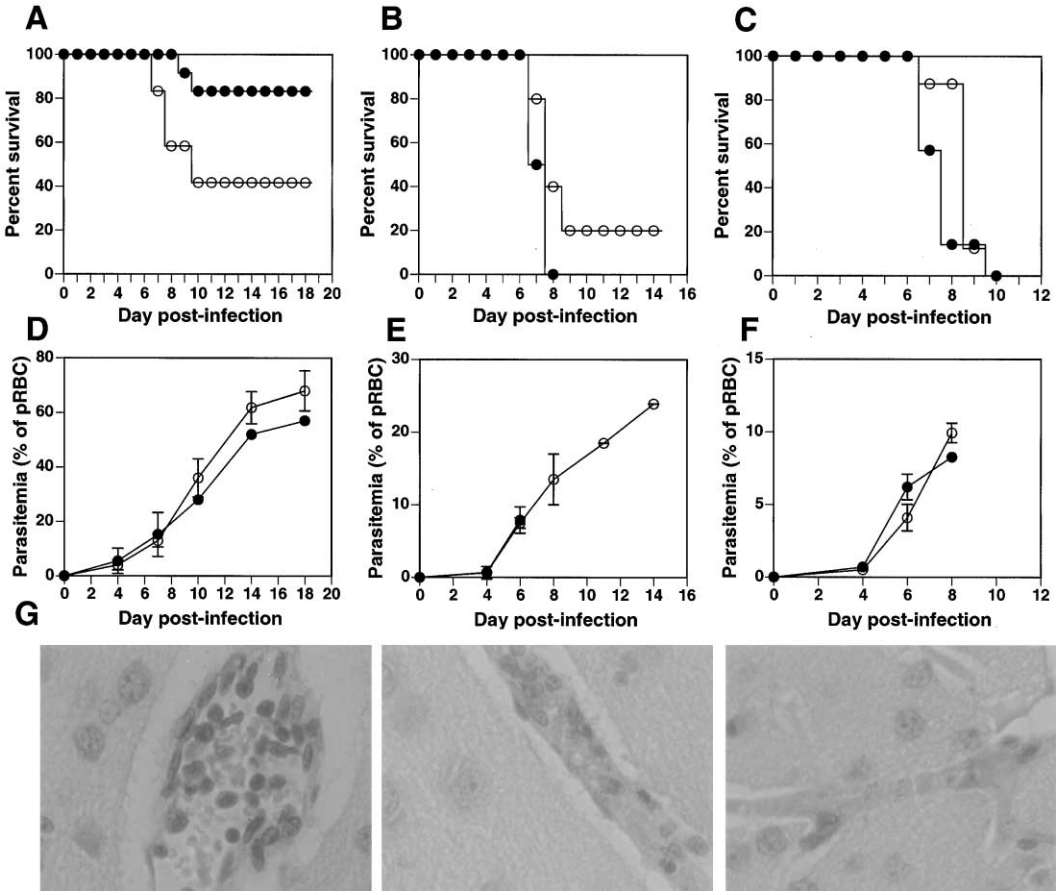


Figure 1. CD1d-Restricted NKT Cells Partially Control Resistance and Susceptibility of Mice to Experimental Cerebral Malaria

WT (closed circles) or mutant mice (open circles) were infected with *P. berghei* ANKA. The percentage survival of BALB/c and CD1$^{-/-}$ BALB/c mice (A), C57BL6 and CD1$^{-/-}$ C57BL6 mice (B), and C57BL6 and Jα281$^{-/-}$ C57BL6 mice (C) was monitored daily. Parasitemia of BALB/c and CD1$^{-/-}$ BALB/c mice (D), C57BL6 and CD1$^{-/-}$ C57BL6 mice (E), and C57BL6 and Jα281$^{-/-}$ C57BL6 mice (F) was assessed from Giemsa-stained blood films. Each point represents the mean parasitemia $\pm$SE of the surviving animals. Each infection is representative of at least four separate experiments. $p < 0.02$, < 0.03, and < 0.05 for (A), (B), and (C), respectively, by Cox-Mantel logrank transformation. (G) Histological examination of brains from C57BL6 WT (left panel), CD1$^{-/-}$ BALB/c (center panel), and BALB/c WT mice (right panel) infected with *P. berghei*. Magnification, $\times$400.

Results

The CD1d/NKT Cell Pathway Partially Controls Resistance and Susceptibility of Mice to Experimental Cerebral Malaria

To investigate the contribution of CD1d-restricted NKT cells to the pathogenesis of experimental cerebral malaria, mice lacking CD1.1 and CD1.2 (CD1$^{-/-}$) on both the disease-resistant BALB/c and disease-susceptible C57BL6 backgrounds, and wild-type (WT) controls, were challenged with *P. berghei* ANKA, and the course of disease was monitored. Unlike BALB/c WT mice, BALB/c CD1$^{-/-}$ mice developed cerebral malaria ($p < 0.02$), and 60% of the animals died between days 7 to 10 postinfection (p.i.) (Figure 1A). This CD1d deficiency associated with increased susceptibility to disease suggests that in BALB/c mice the CD1/NKT pathway provides protection against cerebral malaria. In marked contrast, CD1d-restricted NKT cells appear to play a modest role in promoting disease in C57BL6 mice, as lack of CD1 partially but significantly ($p < 0.03$) protected against cerebral malaria in this genetic background (Figure 1B). C57BL6 Jα281$^{-/-}$ mice lack the invariant Vα14 T cell receptor (TCR) chain and therefore are specifically deficient in Vα14^{+} NKT cells (Cui et al., 1997). When these mice were challenged with *P. berghei* ANKA, they also showed a significant ($p < 0.05$) delay in the disease onset compared to WT controls (Figure 1C), validating the observations in an independent mutant system and demonstrating a role for the Vα14^{+} NKT population in promoting the disease susceptibility of C57BL6 mice. Parasitemia levels were not affected by deletion of CD1 or Jα281 in either genetic background (Figures 1D–1F). Therefore, control of malarial fatalities by CD1d-restricted NKT cells does not operate through effects on parasite growth rates.

The diagnoses of cerebral malaria were confirmed by histological examination of brains taken at various times p.i. Mice dying of cerebral syndrome displayed typical pathology including high levels of vascular occlusion with both parasitized erythrocytes and macrophages (Figure 1G). This pathology was particularly pronounced

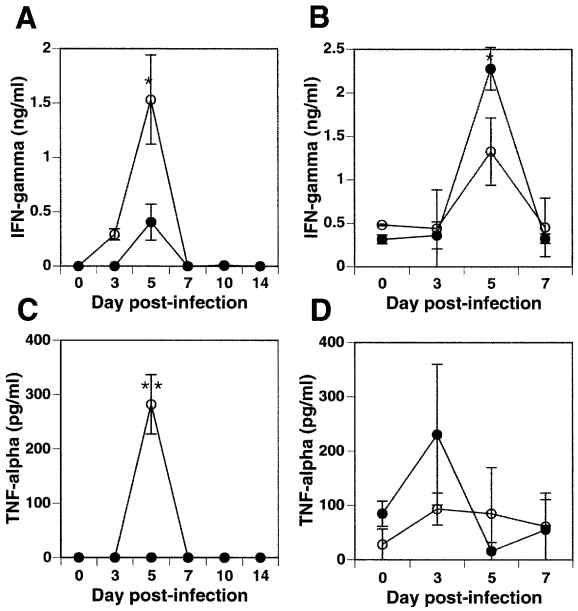


Figure 2. CD1d-Restricted NKT Cells Significantly Control Production of Proinflammatory Cytokines In Vivo in *P. berghei*-Induced Cerebral Malaria

BALB/c (A and C) and C57BL6 (B and D) mice were infected with *P. berghei* ANKA. IFN-γ (A and B) and TNF-α (C and D) levels in sera collected at different time points postinfection from WT (closed circles) or CD1$^{-/-}$ (open circles) mice were measured by capture ELISA. Each experiment is representative of at least three separate infections. Each point represents the mean ±SE. * $p < 0.05$, ** $p < 0.01$ between WT and CD1$^{-/-}$ mice.

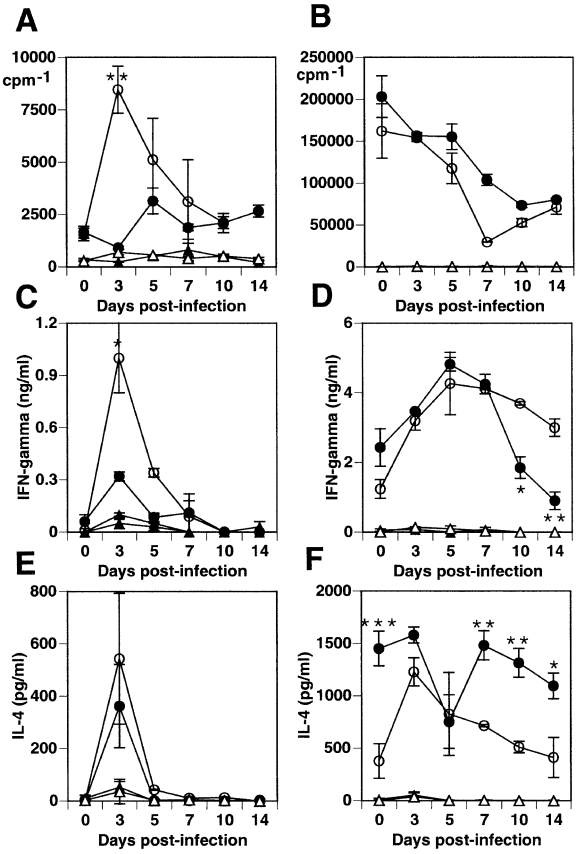


Figure 3. The CD1/NKT Pathway Influences the T_H1/T_H2 Polarization in Response to Malaria

CD4$^+$ T cells from *P. berghei* ANKA-infected BALB/c WT (closed circles) or CD1$^{-/-}$ (open circles) mice were stimulated for 3 days with *P. berghei* ANKA total lysate (A, C, and E) or anti-CD3 (B, D, and F). Cells from WT (closed triangles) and CD1$^{-/-}$ animals (open triangles) were cultured in medium alone as background controls. Cell proliferation was determined by [methyl-^{3}H]-thymidine incorporation (A and B), and IFN-γ (C and D) and IL-4 (E and F) levels in cell culture supernatant by capture ELISA. Each experiment is representative of at least three separate infections. Each point represents the mean of three samples ±SE. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ between stimulated cells from WT and CD1$^{-/-}$ mice.

in C57BL6 mice and was also present, although with reduced severity, in BALB/c CD1$^{-/-}$ animals. BALB/c WT mice in contrast showed absent or very much reduced vascular occlusion despite similar parasite burdens (Figure 1G). Thus, the presence and severity of histological markers of cerebral disease correlate well with the overall fatality rates in these groups.

The CD1d/NKT Cell Pathway Controls Production of Proinflammatory Cytokines In Vivo in Murine Cerebral Malaria

TNF-α and IFN-γ are two proinflammatory cytokines associated with the development of murine cerebral malaria (Grau et al., 1987, 1989). To study whether CD1d-restricted NKT cells regulate the serum levels of these two cytokines in experimental cerebral malaria, sera were collected from BALB/c and C57BL6 WT or CD1$^{-/-}$ mice at different time points p.i., and the cytokine levels were assessed by capture ELISA. Disease-resistant BALB/c WT mice had negligible levels of serum TNF-α and IFN-γ throughout the course of infection (Figures 2A and 2C). In contrast, TNF-α and IFN-γ levels were significantly higher in more susceptible BALB/c CD1$^{-/-}$ mice at day 5 p.i. (Figures 2A and 2C). IFN-γ was lower in the more resistant CD1$^{-/-}$ C57BL6 as compared with fully susceptible WT C57BL6 mice (Figure 2B). Although showing a trend to reduced TNF-α levels in CD1$^{-/-}$ C57BL6 mice, these results were not significantly different (Figure 2D). Thus, CD1d-restricted NKT cells appear to regulate systemic levels of two proinflammatory cytokines associated with murine malarial pathogenesis, with opposing activities depending on the genetic background of the host.

The CD1d/NKT Cell Pathway Controls the T_H1/T_H2 Balance in Response to Malarial Infection

Based on rapid and high output of IL-4 and IFN-γ, the CD1/NKT pathway has been proposed to influence the regulation of T_H1/T_H2 immune responses (Porcelli, 1995). To investigate whether the differential susceptibility to cerebral malaria in BALB/c and C57BL6 mice reflected the ability of CD1d-restricted NKT cells to influence the T_H1/T_H2 balance in response to infection, we examined cytokine production by CD4$^+$ cells isolated from infected WT and CD1$^{-/-}$ mice. At early stages of the infection, CD4$^+$ cells from mice with increased susceptibility to disease, such as CD1$^{-/-}$ BALB/c, proliferated strongly in response to parasite antigen (Figure 3A) whereas

$CD4^+$ cells from WT mice did not proliferate above background controls. $CD4^+$ cells from $CD1^{-/-}$ mice secreted significantly higher levels of IFN-γ at day 3 p.i. (Figure 3C) compared with disease-resistant BALB/c WT controls. No significant differences between BALB/c WT or $CD1^{-/-}$ mice were detected in the parasite-specific IL-4 production (Figure 3E). After day 7 postinfection, the parasite-induced cell death among antigen-specific lymphocytes reported previously (Hirunpetcharat and Good, 1998) results in a depleted and apparently anergic T cell response, and antigen-specific cytokine and proliferative responses become difficult to elicit in vitro.

To investigate the impact of CD1d-restricted NKT cells on global T cell responses, $CD4^+$ cells from BALB/c WT and $CD1^{-/-}$ mice were stimulated with anti-CD3 antibody. Proliferative responses decreased as the infection developed (Figure 3B), indicating progressive T cell anergy associated with malaria infection. $CD4^+$ cells from both WT and $CD1^{-/-}$ BALB/c mice produced high levels of IFN-γ in response to anti-CD3 antibody during the initial stages of the infection. From day 7 onward, IFN-γ levels decreased in BALB/c WT but remained high in $CD1^{-/-}$ mice (Figure 3D). In contrast, IL-4 production was initially downregulated but markedly increased from day 7 postinfection, demonstrating a switch from T_H1 to T_H2 immune response in BALB/c WT mice. This initial downregulation during early time points followed by recovery of IL-4 production was a reproducible feature of infection in several replicate experiments. Interestingly, in the more susceptible BALB/c $CD1^{-/-}$ mice, IFN-γ production was not downregulated, and no switch to IL-4 production was observed (Figures 3D and 3F), indicating that BALB/c CD1d-restricted NKT cells provide help for the development of T_H2 immune responses.

In contrast to BALB/c mice, CD1d-restricted NKT cells promote both proliferative responses and IFN-γ production in WT C57BL6 mice, as partially protected C57BL6 $CD1^{-/-}$ mice showed reduced antigen-specific (Figure 4A) as well as anti-CD3 mediated (Figure 4B) proliferative responses and reduced IFN-γ levels at early time-points p.i. compared to WT animals (Figures 4C and 4D). No significant differences in IL-4 output were found between $CD4^+$ cells from C57BL6 WT or $CD1^{-/-}$ mice (Figures 4E and 4F).

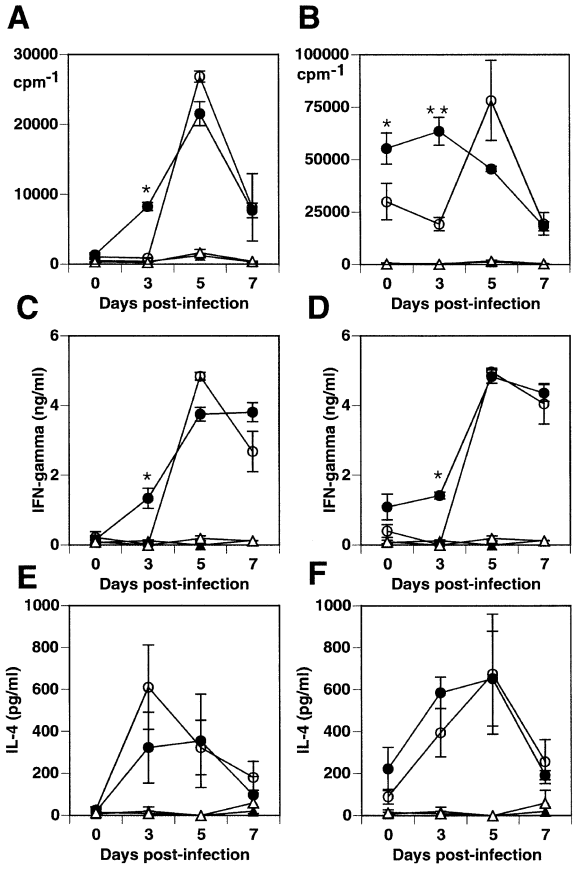
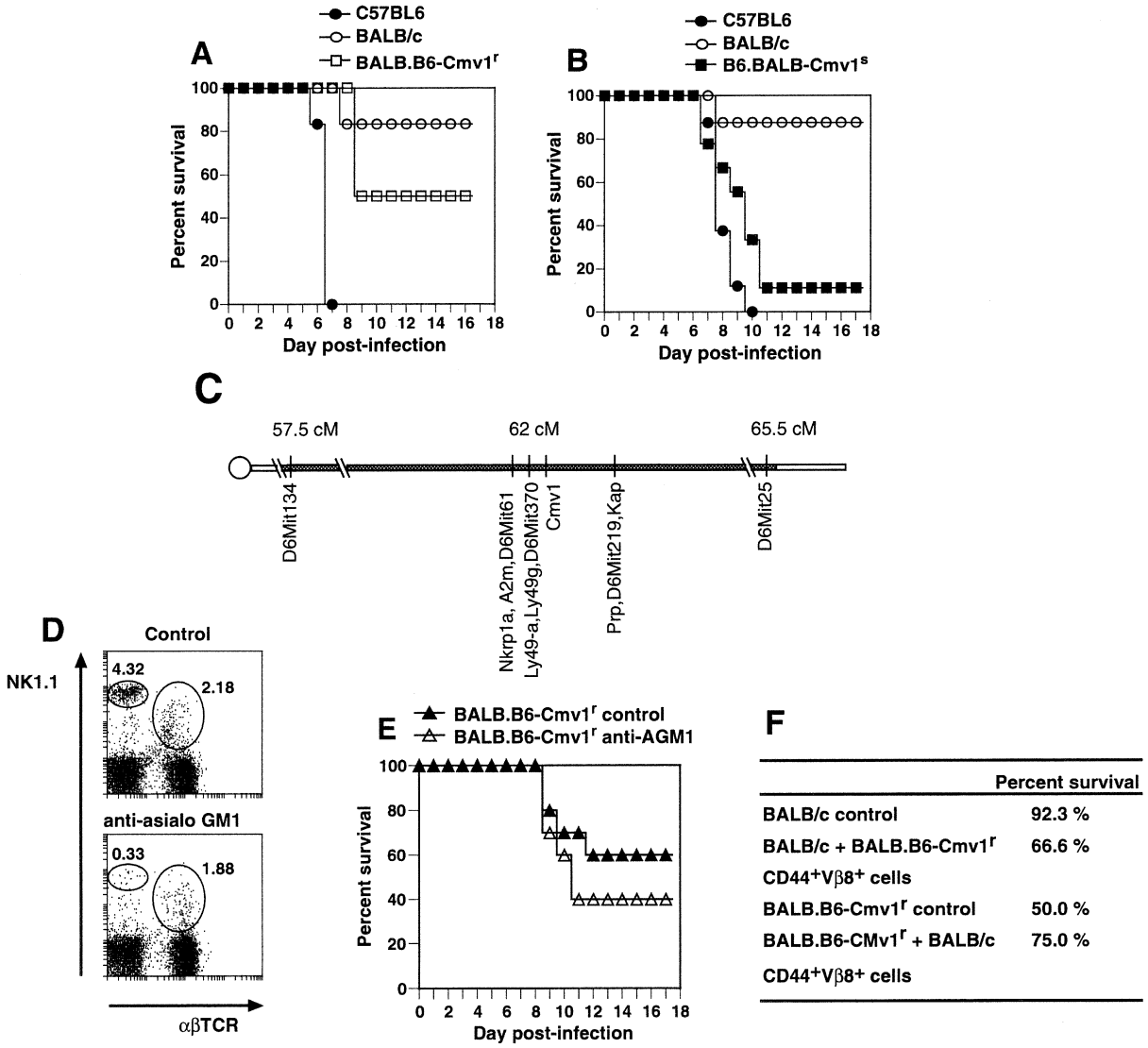


Figure 4. The CD1/NKT Pathway Influences the T_H1/T_H2 Polarization in Response to Malaria

$CD4^+$ T cells from *P. berghei* ANKA-infected C57BL6 WT (closed circles) or $CD1^{-/-}$ (open circles) mice were stimulated for 3 days with *P. berghei* ANKA total lysate (A, C, and E) or anti-CD3 (B, D, and F). Cells from WT (closed triangles) and $CD1^{-/-}$ animals (open triangles) were cultured in medium alone as background controls. Cell proliferation was determined by [methyl-^{3}H]-thymidine incorporation (A and B), and IFN-γ (C and D), and IL-4 (E and F) levels in cell culture supernatant by capture ELISA. Each experiment is representative of at least three separate infections. Each point represents the mean of three samples ±SE. * $p < 0.01$, ** $p < 0.005$ between stimulated cells from WT and $CD1^{-/-}$ mice.

Control of Malarial Pathogenesis by the Natural Killer Complex

Figures 1–4 indicate that the regulatory role of CD1-restricted NKT cells differs markedly between BALB/c and C57BL6 mice. Both NK and NKT cells from C57BL6 and BALB/c mice are known to differ in expression of loci encoded by the NKC (Scalzo et al., 1999). Located on mouse chromosome 6, the NKC comprises several genes (e.g., *Cd69* and *Cd94*) and multigene families (*Nkrp1*, *Nkg2*, and *Ly49*) involved in the activation and inhibition of NK cells. All NKC genes described so far encode type II integral membrane proteins with C-lectin domains having inhibitory or activation activity on cytokine production depending on the presence or absence of immunoreceptor tyrosine-based inhibitory motifs in their intracellular domains. Therefore, NKC products are candidate regulators of malarial pathogenesis. To study whether the NKC genotype can influence the susceptibility to cerebral malaria we made use of NKC homozygous congenic BALB.B6-Cmv1^r mice, in which the region of chromosome 6 containing the NKC from C57BL6 has been introduced onto the BALB/c background (Figure 5C). We also utilized the reverse congenic B6.BALB-Cmv1^s mice, in which C57BL6 background animals bear a 1.5 cM region expressing the NKC of BALB/c mice (Scalzo et al., 1995, 1999). Unlike BALB/c WT mice, BALB.B6-Cmv1^r congenic animals which express the C57BL6 NKC developed cerebral malaria, and 50% of the animals died between days 7 and 9 p.i. In contrast, B6.BALB-Cmv1^s mice were partially protected against disease compared to C57BL6 WT mice (Figures 5A and 5B). Parasitemia levels were not significantly different between WT and congenic mice (data not shown). In addition, histological examination of brains taken from BALB.B6-Cmv1^r animals revealed a typical histological profile consisting of cerebral vasculature occluded with



	Percent survival
BALB/c control	92.3 %
BALB/c + BALB.B6-Cmv1^r CD44$^+$Vβ8$^+$ cells	66.6 %
BALB.B6-Cmv1^r control	50.0 %
BALB.B6-CMv1^r + BALB/c CD44$^+$Vβ8$^+$ cells	75.0 %

Figure 5. Control of Malarial Pathogenesis by the NKC

(A and B) Groups of 10–12 BALB/c, C57BL6, BALB.B6-Cmv1^r, and B6.BALB-Cmv1^s mice were infected with *P. berghei* ANKA. The percentage survival was monitored daily.

(C) Map of the distal region of mouse chromosome 6 in the BALB.B6-Cmv1^r mouse strain. The region inherited by the congenic mice from the C57BL6 background is indicated in gray.

(D) BALB.B6-Cmv1^r mice were depleted of NK cells by i.v injection with anti-asialo GM1 antibody. NK cell depletion was confirmed by FACS.

(E) Groups of ten anti-asialo GM1-treated and control BALB.B6-Cmv1^r mice were infected with *P. berghei* ANKA and percentage survival was monitored daily.

(F) BALB/c mice were injected i.v. with 6.5 $\times$ 10^5 CD44$^+$Vβ8$^+$ cells (sorted as shown in Figure 7A) from malaria-infected BALB.B6-Cmv1^r animals, and BALB.B6-Cmv1^r mice received CD44$^+$Vβ8$^+$ cells from malaria-infected BALB/c wild-types. Adoptively transferred mice and controls were then challenged with *P. berghei* ANKA. The percentage survival was monitored daily.

both parasitized erythrocytes and macrophages as found previously in BALB/c CD1$^{-/-}$ mice (data not shown). Thus, the NKC is a significant determinant of murine cerebral malaria and, in common with the CD1d/NKT pathway, imparts either protective or disease-enhancing properties depending on genotype.

NKC loci are expressed on NK cells as well as NKT cells. To determine which population was responsible for mediating NKC-dependent regulation of malarial pathogenesis, BALB.B6-Cmv1^r mice were treated with anti-asialoGM1 antibody, known specifically to deplete NK cells without affecting NKT cells (Smyth et al., 2001). Depletion of NK cells in BALB.B6-Cmv1^r mice, confirmed by FACS analysis (Figure 5D), did not reverse their increased susceptibility to cerebral malaria ($p > 0.38$) (Figure 5E). To further investigate whether the expression of NKC markers influences the regulation of malarial pathogenesis by NKT cells, CD44$^+$Vβ8$^+$ NKT cells from BALB.B6-Cmv1^r or BALB/c infected donors (purified by FACS sorting) were adoptively transferred into naive recipient mice. CD44$^+$Vβ8$^+$ cells from BALB.B6-Cmv1^r mice transferred into BALB/c recipients

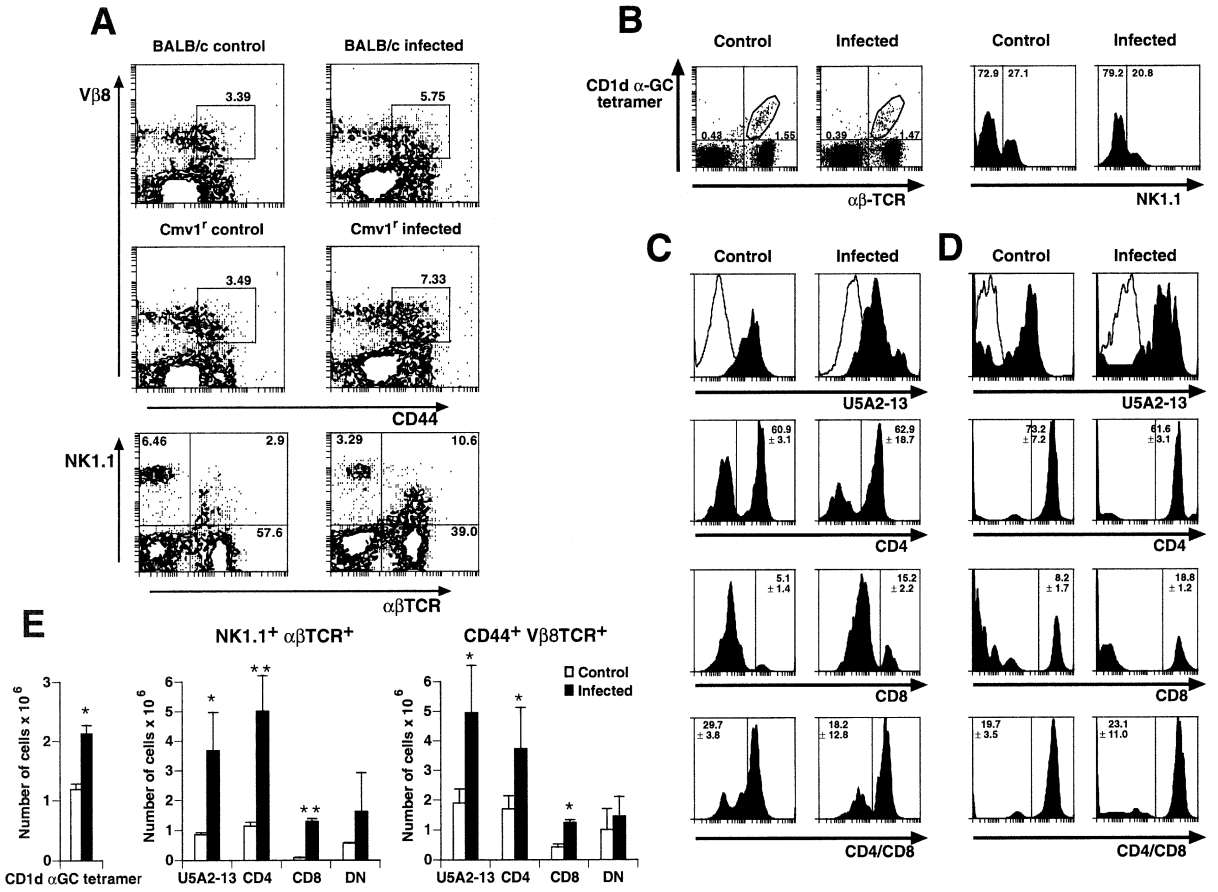


Figure 6. Expansion of Splenic NKT Cells in Response to *P. berghei* ANKA Infection

(A) Spleen cells from naive or infected BALB/c and BALB.B6-Cmv1^r mice were stained with anti-CD44 and anti-Vβ8TCR antibodies (top and center panels). BALB.B6-Cmv1^r splenocytes were also stained with anti-NK1.1 and anti-TCRαβ antibodies (bottom panel).

(B) BALB.B6-Cmv1^r splenocytes were stained with CD1d α-GalCer tetramer and anti-TCRαβ antibodies. The expression of NK1.1$^+$ cells was analyzed in the double-positive population indicated.

(C and D) BALB.B6-Cmv1^r splenocytes were stained with anti-NK1.1 and anti-TCRαβ antibodies (C) or with anti-CD44 and anti-Vβ8TCR antibodies (D). The expression of U52A-13, CD4, CD8, and CD4/CD8 positive cells was analyzed in the double-positive populations (shaded histograms). Empty histograms represent staining in gated NK1.1$^-$ or Vβ8$^-$ T cells.

(E) The absolute number α-GalCer tetramer$^+$-TCRαβ$^+$ and NK1.1$^+$TCRαβ$^+$ or CD44$^+$Vβ8$^+$ subsets in the spleens of malaria infected or naive BALB.B6-Cmv1^r mice. Each bar represents the mean of three to five mice ±SE. * $p < 0.05$, ** $p < 0.005$ between cells from infected mice and controls.

increased their susceptibility to *P. berghei*-mediated cerebral malaria (Figure 5F). In contrast, CD44$^+$Vβ8$^+$ cells from malaria-infected BALB/c mice were able to transfer partial resistance to BALB.B6-Cmv1^r animals (Figure 5F). As predicted, the reversal of phenotypes was partial, due possibly to the competing contribution of resident NKT cells in recipients. Thus, NKC expression on NKT cells may be responsible for the increased susceptibility to disease of BALB.B6-Cmv1^r mice.

A Heterogeneous NK1.1$^+$ αβTCR$^+$ Population Expands in Response to *P. berghei* Infection

We sought to determine whether NKT cells were expanded in response to *P. berghei* infection. Figure 6A shows a 70% and 100% increase in the percentage of Vβ8$^+$CD44$^+$ cells in the spleens of BALB/c and BALB.B6-Cmv1^r mice, respectively, at day 3 p.i. The percentage of NK1.1$^+$αβTCR$^+$ cells increased greatly in the spleens of infected BALB.B6-Cmv1^r mice (to 10.6%) compared to noninfected (2.9%) control animals (Figure 6A) reaching a peak at day 5 p.i. To undertake further phenotyping, splenocytes from BALB.B6-Cmv1^r naive and infected (day 5 p.i.) mice were stained with various antibodies and with CD1d α-Galactosylceramide (α-GalCer) tetramers. The absolute number of CD1d α-GalCer tetramer$^+$ αβTCR$^+$ NKT cells doubled in response to malarial infection (Figure 6E). The relative proportion of $^+$αβTCR$^+$ CD1d α-GalCer tetramer$^+$ cells, however, did not change in spleens of infected mice compared to controls (Figure 6B). Interestingly, although splenic CD1d α-GalCer tetramer$^+$ cells were expanded, the proportion of these cells that were NK1.1$^+$ was slightly reduced in infected animals (Figure 6B). We also gated NK1.1$^+$αβTCR$^+$ (Figure 6C) or Vβ8$^+$CD44$^+$ cells (Figure 6D) from naive and infected BALB.B6-Cmv1^r mice (day 5 p.i) and examined expression of the pan NK-

NKT cell marker U5A2-13, CD4, and CD8. The relative proportions of U5A2-13$^+$ (60%–80%), CD4$^+$ (60%–70%), and DN cells (20%–30%) were similar in spleens from naive and infected mice (Figures 6C and 6D). However, the absolute number of these NKT cell subsets substantially increased in response to infection (Figure 6E). Both the percentage and absolute number of CD8$^+$ cells among the NK1.1$^+\alpha\beta$TCR$^+$ and Vβ8$^+$CD44$^+$ cells were significantly higher in infected compared to noninfected control mice (Figures 6C–6E), although this expansion was not alone sufficient to account for the absolute increase in these populations. CD8 T cells, carrying the TCR Vβ8$^+$, have been previously described to expand in response to malaria infection (Boubou et al., 1999). Taken together, these results indicate that a heterogeneous NK1.1$^+\alpha\beta$TCR$^+$ population including conventional and nonconventional NKT cells as well as CD8 T lymphocytes expands in response to malaria infection.

The NKC Phenotype Influences the Capacity of NKT Cells to Secrete Cytokines

To determine whether NKC phenotype can directly influence the capacity of NKT cells to secrete cytokines, Vβ8$^+$CD44$^+$ NKT cells were isolated by FACS sorting from splenocytes of BALB/c, C57BL6, and BALB.B6-Cmv1^r mice (Figure 7A). The cells were then stimulated for 4 days with plate-bound anti-CD3 or anti-NK1.1 antibodies. When sorted Vβ8$^+$CD44$^+$ cells were cultured with anti-CD3, they secreted high levels of both IL-4 and IFN-γ (Figures 7B and 7C). Stimulation with anti-NK1.1 antibody induced only IFN-γ production but no IL-4 in NKT cells from C57BL6 and BALB.B6-Cmv1^r mice (Figures 7B and 7C). Thus, stimulation through the TCR results in comparable levels of both IFN-γ and IL-4 output in BALB/c and C57BL6 mice. However, activation of NKC receptors such as NK1.1 expressed in the C57BL6 background preferentially induces IFN-γ production.

To investigate whether the NKC phenotype also influences the ability of NKT cells to secrete cytokines in response to malaria infection, sorted Vβ8$^+$CD44$^+$ (Figure 7A) cells from *P. berghei*-infected BALB/c and BALB.B6-Cmv1^r mice were stimulated with α-GalCer, and proliferative responses and IL-4 and IFN-γ production were analyzed. Vβ8$^+$CD44$^+$ cells from both BALB/c and BALB.B6-Cmv1^r mice proliferated in response to α-GalCer, confirming the presence of Vα14$^+$ NKT cells within that population (Figure 7D). In both mouse strains, cell proliferation decreases as the infection develops, suggesting that CD1d-restricted NKT cells do not evade the progressive T cell anergy associated with malaria infection. Like cell proliferation, cytokine output gradually decreases in response to infection. α-GalCer-stimulated NKT cells from both infected BALB/c and BALB.B6-Cmv1^r mice produced similar levels of IL-4 (Figure 7E). By days 7 and 10 p.i., however, BALB/c NKT cells cease IFN-γ production almost entirely. In contrast, NKT cells from BALB.B6-Cmv1^r mice continued to secrete around 15 times more IFN-γ than NKT cell from BALB/c mice (Figure 7F). The ex vivo cytokine production by NKT cells from infected mice was also analyzed. To that end, splenocytes from infected mice were stained with anti-CD44 and anti-Vβ8, and intracellular IL-4 and IFN-γ were analyzed by FACS on gated CD44$^+$Vβ8$^+$. CD44$^+$Vβ8$^+$cells from BALB.B6-Cmv1^r mice displayed nearly three times higher IFN-γ levels than cells from BALB/c mice (Figure 7G). IL-4 responses were in general low, and no differences were found between BALB/c and BALB.B6-Cmv1^r mice. Similar results were obtained when intracellular cytokines were analyzed on gated U5A2-13$^+$TCR$^+$ cells (data not shown). Thus, in malaria infection, expression of C57BL6 NKC markers predisposes NKT cells to increased IFN-γ production. These data are highly concordant with the proposition that differential expression of NKC loci may account in part for the differential immunological behavior of the CD1/NKT cell pathway in C57BL6 and BALB/c mice.

Discussion

This study shows that CD1d-restricted NKT cells influence the T_H1/T_H2 balance, pathogenesis, and fatality in murine cerebral malaria, and cells from distinct genetic backgrounds appear to have opposing properties in this regard. The CD1/NKT cell pathway appears to favor the development of T_H2 polarization and resistance to cerebral malaria in BALB/c mice, and induces early IFN-γ production and promotes pathology in susceptible C57BL6 animals. CD1d-restricted NKT cells clearly influence the overall production of IFN-γ and TNF-α in vivo during malaria infection. The differential expression of molecules encoded by the NKC may account in part for the opposing roles of NKT cells in C57BL6 and BALB/c mice in response to *P. berghei* infection. Our data, moreover, indicate that the NKC is a significant genetic determinant of murine cerebral malaria, imparting partial protection or susceptibility depending on genotype.

Throughout this study, the most pronounced effects in relation to both CD1d-restricted NKT cells and the NKC in protection against cerebral malaria were observed on the BALB/c background, compared with a smaller but significant and reproducible effect in promoting disease susceptibility and T_H1 responses in C57BL6 mice. This differential penetrance presumably reflects a relatively greater contribution by other susceptibility loci in the latter case, i.e., it seems likely that additional pathways or loci promote disease susceptibility in C57BL6 mice.

Previous studies in rodent malaria infections show that an initial T_H1 response switches after the first week to a T_H2 response (Langhorne et al., 1989; Taylor-Robinson et al., 1993). The sequential onset of T_H1-mediated cellular mechanisms and T_H2-mediated antibody responses is proposed to be necessary for the immunological control of *Plasmodium* blood stage infections. Our data indicate that a similar process occurs in *P. berghei*-infected disease-resistant BALB/c mice and that this is regulated by CD1d-restricted NKT cells. In this case, the switch from T_H1 to T_H2 immune responses may downregulate the strong proinflammatory response involved in the cerebral syndrome, explaining a protective role against disease for CD1d-restricted NKT cells in BALB/c mice. These findings from an infectious disease are consistent with data from a T_H1-driven autoimmune condition, where adoptive transfer or overexpres-

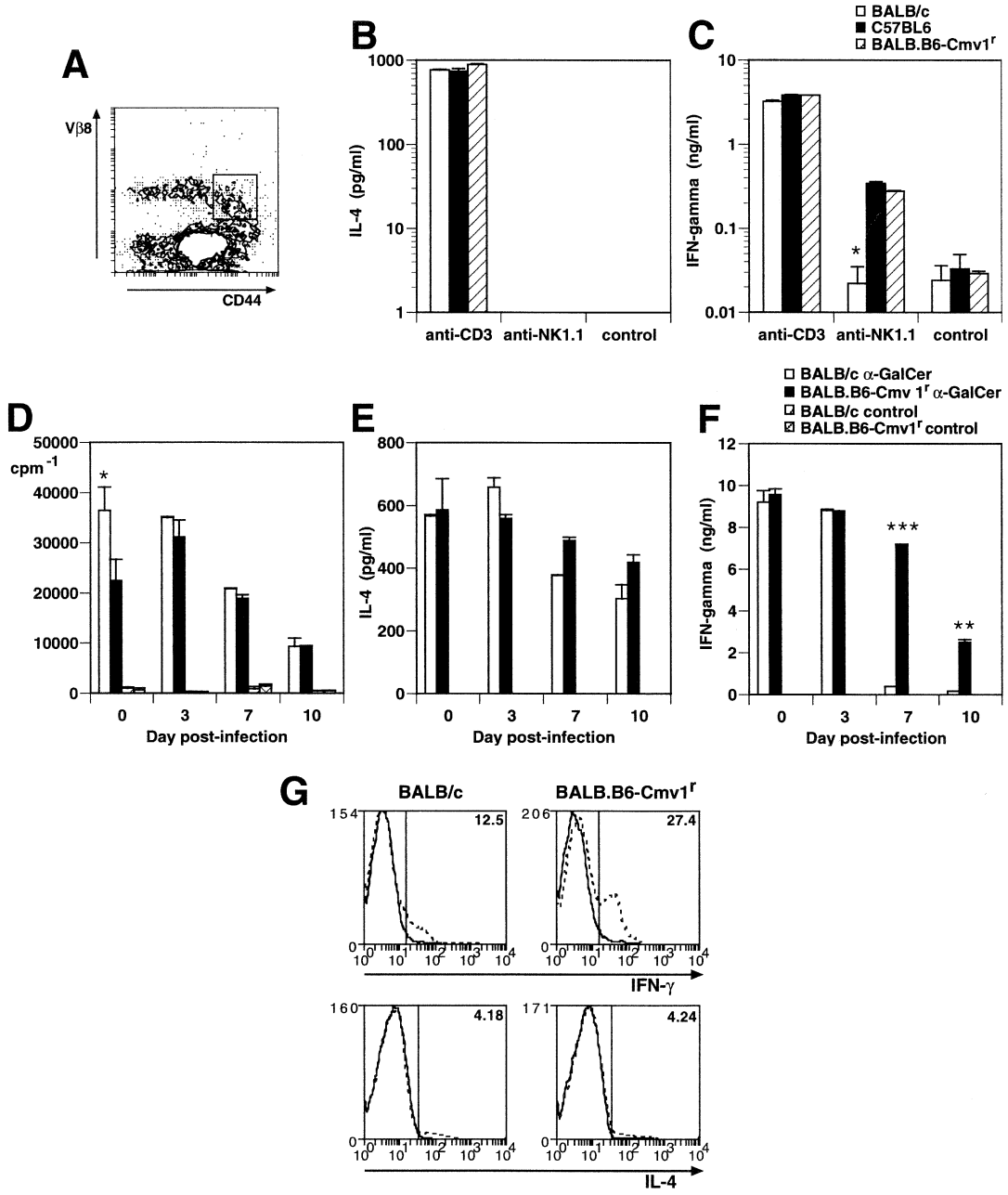


Figure 7. The NKC Phenotype Influences the Capacity of NKT Cells to Secrete Cytokines

(A) The Vβ8$^+$CD44$^+$ population of splenic NKT cells was purified by FACS sorting from splenocytes of BALB/c, C57BL6, and BALB.B6-Cmv1^r mice. The purified NKT cells were stimulated for 4 days with plate-bound anti-CD3, anti-NK1.1, or medium alone.

(B and C) IFN-γ (B) and IL-4 (C) levels were measured in cell culture supernatants by ELISA. Each bar represents the mean of three samples ±SE. * $p < 0.05$ between BALB/c and C57BL6 or BALB.B6-Cmv1^r mice.

(D–F) Vβ8$^+$CD44$^+$ NKT cells from *P. berghei*-infected BALB/c and BALB.B6-Cmv1^r mice were stimulated with α-GalCer for 4 days. Cell proliferation was determined by [methyl-^{3}H]-thymidine incorporation (D), and IL-4 (E) and IFN-γ (F) levels in cell culture supernatant by capture ELISA. Each bar represents the mean of three samples ±SE. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$ between cells from α-GalCer-stimulated BALB/c and BALB.B6-Cmv1^r mice.

(G) Ex vivo cytokine production by NKT cells. Splenocytes from BALB/c and BALB.B6-Cmv1^r malaria infected-mice were stained with anti-CD44 and anti-Vβ8TCR antibodies, and intracellular IL-4 or IFN-γ was analyzed by FACS. Representative histograms from cells gated on CD44$^+$Vβ8$^+$ are shown. The dotted lines represent cytokine staining from infected mice (day 7), and the full lines show noninfected controls.

sion of NKT cells in nonobese diabetic (NOD) mice protects against diabetes (Hammond et al., 1998; Lehuen et al., 1998). Furthermore, defects in NKT cell number and function occur in murine and human type I diabetes (Godfrey et al., 1997; Wilson et al., 1998), and CD1$^{-/-}$ NOD mice are more susceptible to the disease (Shi et al., 2001).

Genetically determined resistance to murine cyto-

megalovirus (MCMV) is controlled by a gene designated *Cmv1* that regulates MCMV replication in the spleens of mice (Scalzo et al., 1995). C57BL6 mice express the *Cmv1*^{r} allele and are resistant to this condition whereas BALB/c mice express the *Cmv1*^{s} allele and show high viral titers. Generation of congenic mouse strains has allowed the mapping of these alleles to the NKC located on the distal region of mouse chromosome 6. Recent studies show that *Cmv1*^{r} maps to the NK activation receptor Ly49H (Dokun et al., 2001). Molecules encoded by the NKC include different receptors involved in the activation or inhibition of NK cells. They are all type II integral proteins with C-lectin domains. The NK1.1 alloantigen is an activation receptor. Its function is still unknown but it has been shown that stimulation with anti-NK1.1 antibody induces NK cell-mediated cytotoxicity and cytokine production (Karlhofer and Yokoyama, 1991). Furthermore, this report and others (Arase et al., 1996) show that crosslinking of NK1.1 preferentially induces IFN-γ and no IL-4 production by CD1d-restricted NKT cells, raising the possibility that carbohydrate recognition by these cells is involved in upregulation of proinflammatory responses by a TCR-independent pathway. Other multigene families encoded within the NKC include Ly49 and NKG2 receptors. Protein products encoded by these genes are responsible for the recognition of MHC class I molecules on potential target cells and subsequent inhibition or activation of cytotoxic activity (Lanier, 1998). Stimulation of activation receptors such as Ly49D (Mason et al., 2000) and Ly49H (Dokun et al., 2001) leads to IFN-γ secretion. Moreover, a recent study demonstrated that a subset of NKT cells expressing high levels of NKG2d selectively produces IFN-γ and TNF-α (Gumperz et al., 2002). Together, this evidence suggests that expression of NKC markers on NKT cells might provide additional pathways for induction of T_H1-like responses.

Most malaria susceptibility/resistance loci described in humans are involved in the control of parasite replication. In murine systems, a congenic study has defined a role for MHC loci in determining resistance to *P. chabaudi* infection (Wunderlich et al., 1988). Quantitative trait locus (QTL) analyses on genome scans from murine intercrosses identify alleles on chromosomes 8 and 9 (*char1* and *char2*) with 30%–40% penetrance in controlling *P. chabaudi* replication (Foote et al., 1997; Fortin et al., 1997). The present study uses congenic mice to establish one or more NKC loci on mouse chromosome 6 exerting 40%–50% penetrance in the control of *P. berghei*-induced pathogenesis and fatality through mechanisms independent of parasite replication. The murine NKC and human syntenic regions constitute significant regulatory components of innate immunity, exhibiting a high level of functional polymorphisms in both species. This evidence therefore allows formulation of the testable hypotheses that syntenic human chromosomal regions corresponding to *char1*, *char2*, and the NKC may be associated with resistance or susceptibility to pathogenesis and fatalities in human malaria.

Much useful evidence to date concerning NKT cell function in vivo is provided by the exogenous administration of α-GalCer (Kawano et al., 1997). However, this glycolipid antigen stimulates only through the TCR. *P. berghei* ANKA infection induces a generalized activation of the immune system and provides a framework allowing the characterization of the NKT cell function under physiological conditions. This model provides evidence that NKT cells can be stimulated by pathways other than the TCR and that the genetic background of the host influences the regulatory impact of NKT cells on the global immune response. Moreover, this study and other infection models including *Leishmania major* (Ishikawa et al., 2000), Hepatitis B virus (Baron et al., 2002), *Crytococcus neoformans* (Kawakami et al., 2001), and *Salmonella* (Naiki et al., 1999) indicated that NKT cell populations become highly expanded in response to infection. In contrast, stimulation with glycolipids like α-GalCer results in strong TCR-driven NKT cell activation (Kawano et al., 1997), followed by massive NKT cell death and homeostatic replacement from the bone marrow (Eberl and MacDonald, 2000). It is therefore possible that stimulation of NKT cells with physiological ligands expressed during viral, bacterial, or parasitic infections results in alternative or additional activation pathways. Studies aimed at identification and characterization of NKT cell natural ligands are necessary to address this proposition.

In addition to α-GalCer, CD1d-restricted NKT cells recognize GPIs of parasite origin when these agents are supplied as exogenous antigens (Schofield et al., 1999) but not when expressed as endogenous ligands within the antigen-presenting cell (Molano et al., 2000). Malarial GPI comprises greater than 95% of plasmodial carbohydrate, is the main posttranslational modification of parasite antigens (Gowda et al., 1997), and is therefore a reasonable candidate antigenic stimulus for NKT cells in malaria infection. NKT cells may be influenced toward a T_H2 polarized cytokine profile by immunization with α-GalCer (Burdin et al., 1999; Singh et al., 1999). These considerations raise the possibility of reducing susceptibility to severe malarial disease in human populations by immunomodulation via a T_H2-biased, CD1-restricted glycolipid vaccine.

Experimental Procedures

Mice and Infections

Eight- to twelve-week-old BALB/c, BALB/c CD1$^{-/-}$ (F_{10} generation [Smiley et al., 1997]), C57BL6, C57BL6 CD1$^{-/-}$ (F_6 generation [Sonoda et al., 1999]), C57BL6 Jα281$^{-/-}$ (F_9 generation [Cui et al., 1997]), BALB.B6-Cmv1^r (F_6 generation [Scalzo et al., 1995]), and B6.BALBCmv1^s (F_6 generation [Scalzo et al., 1999]) mice were used throughout the study. The BALB.B6-Cmv1^r and B6.BALBCmv1^s mice were backcrossed using speedy marker-assisted congenic mapping methods. Groups of 10–15 BALB/c WT, BALB/c CD1$^{-/-}$, BALB.B6-Cmv1^r, and B6.BALBCmv1^s were injected i.p. with 1×10^6, and C57B/6 WT, C57B/6 CD1$^{-/-}$, and Jα281$^{-/-}$ mice received 1×10^4, *P. berghei* ANKA-infected erythrocytes. In some experiments, mice were injected i.v. with 20 μg of anti-asialo GM1 antibody (Wako, Osaka, Japan) 6 hr before challenge in order to deplete NK cells. NK cell depletion was confirmed by flow cytometry. For adoptive transfer experiments, mice were injected i.v. with 6.5×10^5 CD44$^+$Vβ8$^+$ cells purified from infected mice (day 7 p.i.) by FACS sorting as described below. Control mice received saline. Four hours later, adoptively transferred mice and controls were challenged with *P. berghei* ANKA. Parasitemia was assessed from Giemsa-stained smears of tail blood prepared every 2–3 days. Mortality was checked daily. Mice were judged as developing cerebral malaria if they displayed neurological signs such as ataxia, loss of reflex, and hemiplegia, and died between days 6 to 12 postinfection with relatively low parasitemia. Statistical differences in mortality rates of groups of *P.*

berghei infected mice during this period of susceptibility to cerebral malaria were assessed by Cox-Mantel logrank analysis. All experiments were performed in compliance with local Animal Ethics Committee requirements.

Histology

For histological analysis of cerebral pathology, brains were taken into 10% neutral-buffered formalin, sectioned (5 μm), and stained with hemotoxylin and eosin. Slides were coded and scored blind for histological evidence of cerebral syndrome.

Lymphoproliferative Assays

Spleen cells from BALB/c and C57BL6, WT and CD1$^{-/-}$ mice (n = 3) were collected at different times postinfection with *P. berghei* ANKA. Splenic CD4$^+$ cells were purified by positive selection with Dynabeads following the manufacturer's instructions (98%–99% purity) (Dynal Biotech, Norway). For proliferation assays, CD4$^+$ cells suspended in complete RPMI-1640 medium, 5% fetal calf serum, were seeded in 96-well plates at a density of 5×10^5 cells/ml. Naive syngeneic spleen cells irradiated to 3000 rads were added as antigen-presenting cells at a density of 2×10^6 cells/ml. Cells were then stimulated in triplicate for 3 days with *P. berghei* ANKA total lysate (50 μg/ml) or anti-CD3 (5 μg/ml, Pharmingen, San Diego, CA). Cells cultured in medium alone were used as background controls. [Methyl-^{3}H]-thymidine (2 μCi/well, 5 Ci/mmol, Amersham, UK) was added 16 hr before harvest, and radioactivity was measured in a betaplate counter.

ELISA for IL-4, IFN-γ, and TNF-α Detection

The following pairs of antibodies were used: 11B11 for capture and BVD6-24G2 for detection of IL-4; R4-6A2 for capture and XMG1-2 for detection of IFN-γ, and G281-2626 for capture and MP6-XT3 for detection of TNF-α (all antibodies from Pharmingen, San Diego, CA). Antibodies used for detection were biotinylated. Ninety-six-well plates were coated with capture antibody by overnight incubation at 4°C in phosphate buffer (pH 9) for IL-4 and IFN-γ, and phosphate buffer (pH 6) for TNF-α. Plates were then blocked with 1% BSA for 1 hr at 37°C. Splenocyte culture supernatants or sera from infected mice were tested in duplicate by overnight incubation at 4°C under mild agitation. The plates were then incubated for 3 hr at 20°C with the respective biotinylated antibody followed by a 2 hr incubation at 20°C with streptavidin-peroxidase conjugate (Pierce, Rockford, IL). Bound complexes were detected by reaction with tetramethylbenzidine (KBL, Gaithersburg, MD) and H_2O_2. Absorbance was read at 450 nm. The cytokine concentration in samples was calculated as pg/ml using recombinant murine cytokines (Pharmingen, San Diego, CA) for the preparation of standard curves.

Flow Cytometry

Spleen cells from BALB/c and BALB.B6-Cmv1^{-r} mice were incubated with anti-CD16 antibody (Fc-block), washed, and stained with NK1.1-PE or FITC-conjugated together with anti-αβTCR-FITC or CyChrome-conjugated antibodies for 1 hr on ice. NKT cells were also characterized by staining with anti-CD44-PE or CyChrome-conjugated and anti-Vβ8 TCR-FITC-conjugated antibodies. In some experiments cells were stained with additional antibodies, i.e., FITC- or PE-conjugated anti-CD4, FITC-, or PE-conjugated anti-CD8, PE-conjugated U5A2-13 (all antibodies from Pharmingen, San Diego, CA), and CD1d α-GalCer tetramer (kind gift of Dr. M. Kronenberg). The cells were then washed two times with PBS containing 1% FCS and suspended in 200 μl of PBS. For intracellular cytokine staining, spleen cells from infected mice were incubated with Fc-block, washed, and stained with surface markers as described above. The cells were fixed and permeabilized with Citofix/Citoperm (Pharmingen, San Diego, CA), and incubated with anti-IFN-γ-FITC or anti-IL-4-PE conjugated antibodies. Appropriate isotype-matched controls were included. The stained cells were then analyzed by FACScan. Dead cells were gated out by forward and side scatter.

Purification and Stimulation of Splenic NKT Cells

Spleen cells from naive or *P. berghei*-infected BALB/c, C57BL6, and BALB.B6-Cmv1^{-r} mice were incubated with anti-CD16 antibody washed and stained with anti-CD44-PE conjugated antibody and anti-Vβ8 TCR-FITC-conjugated antibody (all antibodies from Pharmingen, San Diego, CA) for 1 hr on ice. After washing two times with PBS, double-positive cells were isolated by flow cytometry. The purified cells were seeded in triplicates in 96-well plates at a density of 3×10^4 cells and stimulated with plate-bound anti-CD3 (10 μg/ml), plate-bound anti-NK1.1 (100 μg/ml), or with α-GalCer, (50 ng/ml) for 4 days. α-GalCer was kindly provided by the Pharmaceutical Research Laboratories, Kirin Brewery (Gumna, Japan). In α-GalCer-stimulated cultures, naive syngeneic spleen cells depleted of T cells with Dynabeads (Dynal Biotech, Norway) and irradiated to 3000 rads were added as antigen-presenting cells at a density of 2×10^6 cells/ml. Cells cultured in medium alone were used as background controls. [Methyl-^{3}H]-thymidine (2 μCi/well, 5 Ci/mmol, Amersham, UK) was added 16 hr before harvest, and radioactivity was measured in a betaplate counter. The cell culture supernatants were collected to measure the production of IL-4 and IFN-γ by capture ELISA.

Statistic Analysis

A paired-sample Student's t test was used for data evaluation. Differences in mortality rates of *P. berghei*-infected mice during the period of susceptibility were assessed by Cox-Mantel logrank analysis.

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